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Detection of Heat-Stable Enterotoxigenic *Escherichia coli* by Hybridization with an RNA Transcript Probe

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Heat-stable enterotoxigenic *Escherichia coli* was identified by nucleotide hybridization with RNA transcripts of the gene encoding heat-stable A-2 enterotoxin. Radiolabeled enterotoxin gene RNA transcripts are easier to prepare and avoid the preparation of cloned DNA probes that can be nonspecific if they contain cloning vector DNA.

Enterotoxigenic *Escherichia coli* (ETEC) is usually identified by testing isolates for enterotoxin production in bioassays or serological tests (2, 7, 11, 14, 15). Alternatively, ETEC can be identified by detecting the genes encoding these enterotoxins by DNA hybridization, a method which has been particularly useful in detecting ETEC in large numbers of specimens (3-6).

Specific DNA fragments of plasmids containing the cloned enterotoxin genes have been used in DNA hybridization assays to identify ETEC (4, 5). Probes produced by endonuclease digestion must be electrophoretically separated from plasmid-cloning vector DNA to obtain a specific probe and must also be nick translated (9). Nick translation is relatively easy to perform with large DNA fragments, such as the 850-base-pair fragment used as the heat-labile enterotoxin (LT) gene probe, but is more difficult to perform on small DNA probes used to detect the genes encoding heat-stable enterotoxin (STA-1 [154 base pairs] or STA-2 [216 base pairs]).

Ninety-nine percent of *E. coli* that hybridized with the LT probe produced LT, as measured by the Y-1 adrenal cell assay, but only 72% of *E. coli* that hybridized with the STA-2 probe produced ST, as measured by the suckling mouse assay (5). The lack of specificity of the cloned enterotoxin gene probes was due to probes that contained cloning vector DNA. Ninety-eight percent of *E. coli* that hybridized with the ST oligo probe produced ST (6).

To develop a more convenient probe to identify genes encoding STA-2, we cloned the genes encoding STA-2 into pSP64 and used RNA transcripts to detect ST⁺ ETEC by colony hybridization.

Plasmid DNA was isolated from *E. coli* C600(pSLM004) and was digested with *EcoRI*, *HindIII*, and *HpaII* (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) as previously described (10). The endonuclease digestion fragments were separated by electrophoresis on a 1.5% low-melting-temperature agarose gel (FMC Corp., Marine Colloids Div., Rockland, Maine) in Tris-borate buffer, and a 216-base-pair DNA fragment encoding STA-2 was eluted by heat, phenol extracted, and ethanol precipitated (8). This DNA fragment was blunt-end ligated with pSP64 (Promega Biotec, Madison, Wis.) (8). Transformants were examined by colony

hybridization with the STA-2 [α -³²P]DNA probe as previously described (5). Plasmid lysates of these transformants, designated pTR1 to pTR9, were prepared by the method of Birnboim and Doly (1) and were examined by the Southern technique (13) for hybridization with an alkaline phosphatase-conjugated synthetic ST oligonucleotide probe (Molecular Biosystem, San Diego, Calif.) (12).

Transformants pTR2 and pTR4 were digested with *EcoRI*. These DNA digests were then phenol extracted and ethanol precipitated. These preparations of linear DNA were used as templates for RNA transcriptions: 4 μ l of 5 \times RNA transcription buffer (200 mM Tris hydrochloride [pH 7.5], 30 mM MgCl₂, 10 mM spermidine, and 50 mM NaCl); 2 μ l of 100 mM dithiothreitol; 0.7 μ l of an RNase inhibitor (32 U/ml); 4 μ l of 2.5 mM ATP, GTP, and CTP in H₂O; 2.4 μ l of 100 μ M UTP (12 μ M, final concentration); 1 μ l of linearized DNA template (1 μ g/ μ l); 5 μ l of 10 mCi of [α -³²P]UTP per ml; and 2 μ l of riboprobe SP6 RNA polymerase (15 U/ μ l); these were added to a 1.5-ml conical centrifuge tube and incubated for 60 min at 37°C. A 1- μ l quantity of DNase I (10 mg/ml) was then added and incubated for 15 min at 37°C to digest the DNA template. The labeled RNA transcripts were then extracted with phenol and chloroform and precipitated with ethanol. After the labeled RNA transcript was precipitated, it was dissolved in 200 μ l of 0.5 M NaCl in TE (10 mM Tris hydrochloride [pH 7.2]-1 mM EDTA) and separated from free nucleotides by passing the specimen through an NACS PREPAC column (Bethesda Research Laboratories). The radiolabeled RNA was eluted from the column by adding 600 μ l of 2.0 M NaCl in TE.

Eight clinical isolates of *E. coli* were spotted on nitrocellulose filters (Schleicher & Schuell, Inc., Keene, N.H.) layered on MacConkey agar and incubated overnight at 37°C. The filters were removed and treated as previously described (5). Filters were hybridized with radiolabeled RNA transcripts.

The nitrocellulose filters were incubated at 50°C for 1 h in hybridization buffer containing 50% formamide, 6 \times SSC (1 \times SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% sodium dodecyl sulfate, 5 \times Denhardt solution (1 \times is 0.02% bovine serum albumin, 0.02% Ficoll [molecular weight, 400,000], and 0.02% polyvinylpyrrolidone [molecular weight, 360,000]), 200 μ g of denatured calf thymus DNA per ml, 200 μ g of yeast tRNA per ml, and 1 mM EDTA. Hybridizations were performed overnight at 37°C in hybridization solution with 10⁶ cpm of radioactive probe per ml.

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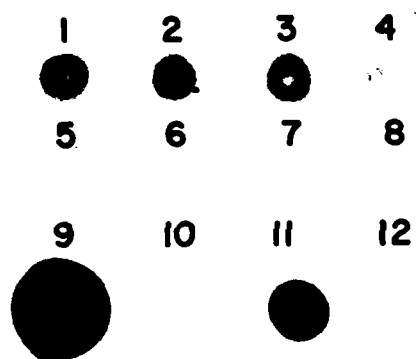


FIG. 1. Colony hybridization of *E. coli* with an [α - 32 P]RNA transcript of pTR2 colonies: 1, *E. coli* OV6 (LT STA-2⁺); 2, *E. coli* OV37 (LT STA-2⁺); 3, *E. coli* OV91 (LT STA-2⁺); 4, *E. coli* OV104 (LT STA-2⁺); 5, *E. coli* E1777 (LT ST⁺); 6, *E. coli* E1780 (LT ST⁺); 7, *E. coli* E1781 (LT ST⁺); 8, *E. coli* E1782 (LT ST⁺); 9, *E. coli* C600(pSLM004); 10, *E. coli* C600(pRIT10036); 11, *E. coli* HB101(pTR2); 12, *E. coli* K-12 Xac.

The filters were then washed three times in $2\times$ SSC at 25°C, treated with 1 μ g of RNase A per ml in $2\times$ SSC at 25°C for 15 min, washed twice in $0.1\times$ SSC-0.1% sodium dodecyl sulfate at 50°C for 30 min, and dried. The filters were exposed to X-Omat X-ray film for 24 h at -70°C. The X-ray films were developed according to the instructions of the manufacturer (Eastman Kodak Co., Rochester, N.Y.).

The cloned STA-2 and ST oligo probes were less sensitive in detecting target cell DNA than in detecting the RNA transcript probe. The STA-2 RNA transcript probe detected 50 pg, the cloned STA-2 probe detected 0.78 ng, and the ST oligonucleotide probe detected 0.39 ng of target cell DNA after hybridization for 16 h and exposure to X-ray film for 16 h at -70°C.

RNA transcripts of pTR2 and pTR4 hybridized with all four LT⁺ ST⁺ ETEC and none of the four non-ETEC. These STA-2 RNA probes also hybridized with *E. coli* C600(pSLM004) but not with *E. coli* K-12 Xac or *E. coli* K-12 C600(pRIT10036) containing the genes encoding STA-1 (Fig. 1).

Synthetic oligonucleotide probes for genes coding for LT and ST have recently become available (6) and eventually may replace cloned DNA fragment probes. However, synthetic probes must either be obtained commercially or made on a DNA synthesizer and then purified by gel electrophoresis or high-pressure liquid chromatography. Specific enterotoxin gene RNA transcription probes offer minimally equipped laboratories an alternative method of identifying ETEC by nucleotide hybridization. Additionally, RNA transcriptions are easier to perform than nick translation reactions and production does not require electrophoresis equipment.

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